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Dissertação especialmente elaborada para obtenção do grau de Mestre em Oncobiologia

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Contents

List of Abbreviations

53BP1	p53 binding protein 1
53BP1-P	53BP1 phosphorylation
ATM	Ataxia-telangiectasia mutated
ATM-P	ATM phosphorylation
ATR	ATM and Rad3-related protein kinase
BRCA1	Breast cancer susceptibility protein 1
BRCA2	Breast cancer susceptibility protein 2
CDK	Cyclin-dependent kinases
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CtIP	Ct-BP interacting protein
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DNA-PK	DNA dependent protein kinase
dNTPs	Deoxyribonucleotide triphosphate
DSB	Double-strand break
EdU	5-ethynyl-2´-deoxyuridine
EMT	Epithelial-to-mesenchymal transition
FUCCI	Fluorescence ubiquitination cell cycle indicator
GFP	Green fluorescent protein
H3K36me3	Histone H3 lysine 36 trimethylation
H	Hours

HR	Homologous Recombination
IMDM	Iscove's Modified Dulbecco's Medium
IR	Ionizing radiation
MHC	Myosin heavy chain
Mrf4	Muscle-specific regulatory factor 4
MRFs	Myogenic regulatory factors
MRN	Mre11-Rad50-Nbs1
Myf5	Myogenic factor 5
MyoD	Myoblast determination protein
MyoG	Myogenin
NCS	Neocarzinostatin
NHEJ	Non-homologous end-joining
PALB2	Partner and localizer of BRCA2
PCNA	Proliferating cell nuclear antigen
PIKK	Phosphatidylinositol 3-kinase related protein kinase
RFP	Red fluorescent protein
RPA	Replication protein A
SSB	Single-strand break
ssDNA	single stranded DNA
UV	Ultra-violet
XLF	XRCCC4-like factor
XRCC4	X-ray cross complementing protein 4
γH2AX	Histone H2AX phosphorylated at Ser139

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Resumo

A integridade da informação genética constitui o fator mais importante do qual a vida depende. A molécula de DNA está constantemente exposta a diferentes tipos de lesões causadas por fontes endógenas, como espécies reativas de oxigénio provenientes do metabolismo, ou fontes exógenas derivadas de fatores mutagénicos, como a luz ultravioleta (UV), radiação ionizante (IR) ou agentes quimioterápico. Estima-se que diariamente as nossas células sofram mais de 100000 alterações espontâneas no DNA, 50 das quais quebras na dupla cadeia (DSB) de DNA.

Independentemente da origem dos fatores mutagénicos eles podem conduzir a variadas alterações na molécula de DNA, tais como inserções, deleções, aductos, intra e intercadeia crosslink. Deste modo, a célula desenvolveu diferentes mecanismos para reparar diferentes tipos de lesões com o objetivo de manter a homeostasia, assegurar a viabilidade celular e promover a sobrevivência. Para que os mecanismos de reparação possam atuar, a célula tem de reconhecer e sinalizar a lesão. A resposta a lesões na molécula de DNA (DDR) é assim constituída pelo reconhecimentos, sinalização e reparação do DNA danificado. As modificações na cromatina representam um importante papel na DDR permitindo a sinalização das lesões, recrutando fatores de reparação e permitindo o acesso da maquinaria de reparação do local de lesão.

Quando a molécula de DNA sofre uma DSB a célula pode adotar diferentes mecanismos para a reparar: reparação homóloga (HR) e a ligação das extremidades não homólogas (NHEJ). Contrariamente à via de HR que requer uma cadeia molde homóloga para restaurar a informação genética, a via de NHEJ conduz à ligação direta das extremidades de DNA da DSB, propiciando deste modo o erro.

Em reposta a lesões na molécula de DNA, os dois primeiros grupos de proteínas a serem recrutados para o local de lesão serão o complexo Mre11-Rad50-NBS1 (MRN) e a família PIKK (*phosphatidylinositol 3-kinase related protein kinase*) onde se inclui ATM (*ataxia-telangiectasia mutated*).O complexo MRN reconhece rapidamente o local da lesão e acumula-se nesse local, recrutando ATM que se autofosforila, permitindo a consequente fosforilação da

variante de histona H2AX (γH2AX). Esta marca espalha-se por toda a região circundante promovendo a ligação de outros fatores de reparação como o MDC1. BRCA1 (*breast cancer susceptibility protein 1*) e 53BP1 (*p53 binding protein 1*) são também recrutados para o local funcionando como mediadores e impulsionadores da sinalização. O fator 53BP1 tem grande importância na determinação da via de reparação a ser adotada, favorecendo desta forma a via NHEJ.

A par destes mecanismos para sinalizar e reparar as lesões na molécula de DNA, a ativação do controlo do ciclo celular é absolutamente essencial para retardar o ciclo celular e providenciar tempo para que a reparação tenha lugar, de modo a impedir que as lesões e consequentes mutações sejam transmitidas às gerações seguintes. No centro desta regulação encontram-se as quinases dependentes de ciclinas (CDK) e as ciclinas. A variação dos níveis de ciclinas por ubiquitinação e degradação no proteassoma ao longo do ciclo celular permite a manutenção das diferentes fases do ciclo celular. A ativação de p53 via Chk2 culmina na ativação de p21, um potente inibidor dos complexos ciclina-CDK, resultando na paragem do ciclo celular.

A reparação as lesões na molécula de DNA em células proliferativas é então mais eficiente por estar disponível o cromatídeo irmão como molde para HR. Tendo em consideração que as células terminalmente diferenciadas podem viver décadas, é então crucial que possuam mecanismos de reparação competentes de modo a prevenir o envelhecimento e a doença. Contudo, muito pouco é conhecido sobre o modo como células pós mitóticas, como os neurónios e miofibras musculares, reparam DSB e ainda qual o papel do ciclo celular e das suas transições em resposta a lesões no DNA em miofibras musculares. Deste modo hipotetizámos que as células musculares terminalmente diferenciadas poderiam reentrar transientemente no ciclo celular em resposta a lesões, com previamente descrito para os neurónios.

Com o objetivo principal de entender os eventos moleculares que têm lugar nos processos de reparação do DNA lesado em células de músculo esquelético e entender as vias de reparação que conduzem à efetiva reparação destas lesões, caracterizámos as principais proteínas ativadas em resposta às lesões induzidas e investigámos a existência de eventuais transições do ciclo

celular que acompanham a reparação do DNA em miotubos, células diferenciadas percursoras de miofibras.

Após a otimização do processo de diferenciação de mioblastos em miotubos, caracterizámos alguns fatores de sinalização envolvidos na DDR. Observámos que, em consequência de da indução de lesões com o radiomimético neocarzinostatina (NCS), a variante de histona H2AX é fosforilada em mioblastos e em miotubos, embora possamos concluir que nos miotubos os seus níveis basais, na ausência de tratamentos com NCS, são mais elevados, evidenciando deste modo uma maior quantidade de lesões endógenas. É ainda evidente que existe reparação das lesões induzidas uma vez que os níveis de γH2AX são repostos para os valores iniciais nos mioblastos e nos miotubos. Observámos também que em resposta às lesões existe uma eficiente ativação de ATM e 53BP1, embora esta seja mais exacerbada e prolongada, para ambas as proteínas, nos miotubos. É ainda observável uma ativação de p21 em resposta às lesões induzidas o que demonstra a ativação do controlo do ciclo celular.

Por forma a avaliar o papel de ATM na fosforilação de H2AX e na ativação de p53/p21, avaliámos a cinética de γH2AX e p53/p21em resposta à indução de lesões com NCS em miotubos após inibição de ATM. Observámos que os níveis de γH2AX. p53 e p21 em resposta a lesões no DNA são significativamente inferiores nas células tratadas com o inibidor de ATM comparativamente com as condição controlo na ausência de inibidor. Este resultado sugere que a DDR em miotubos é dependente de ATM.

A avaliação de formação de *foci* de RAD51 em resposta a lesões no DNA foi conclusiva quanto à incapacidade dos miotubos realizarem a via de HR canónica uma vez que não foi detetado *foci* de RAD51 em miotubos após indução de lesões.

A avaliação das transições do ciclo celular com recurso ao sistema FUCCI (*fluorescence ubiquitination cell cycle indicator*) indiciou que os miotubos não apresentam transições no ciclo celular em resposta a danos no DNA. O estudo da dinâmica de replicação, através da incorporação de EdU, após indução de

lesão também evidenciou que não há replicação de DNA após tratamento com NCS, nem nos mioblastos nem nos miotubos.

Deste modo é evidente que os miotubos não apresentam transições no ciclo celular em resposta a danos no DNA e que a dinâmica de resposta e reparação, bem como as proteínas envolvidas é distinta dos seus progenitores, apontando para que os miotubos não reparem DSB pela via canónica de HR, mas sim pela via NHEJ. Estando descrito que os miotubos são mais resistentes à apoptose do que os seus progenitores, é conclusivo que estas células têm eficientes mecanismos para reparar ou sucumbir as lesões na molécula de DNA.

Com o objetivo de avaliar dinâmica da cromatina durante a reparação do DNA, lesámos um único núcleo num miotubos multinucleado com recurso a radiação laser UV-A e avaliámos morfologicamente as alterações na cromatina. Denotámos, pela análise da redistribuição da histona H2B, que existe uma modulação da arquitetura da cromatina no núcleo lesado em comparação com os núcleos não lesados – condensação da cromatina em resposta a lesão extensa num único núcleo. Hipotetizámos deste modo que a modulação observada poderá ser consequência de um mecanismo de inativação/silenciamento da cromatina no núcleo danificado de modo a não comprometer a sobrevivência de toda a célula e prevenir a transcrição de genes aberrantes. Preliminarmente, a imunofluorescência para deteção da marca epigenética H3K36me3 permitiu constatar uma diminuição dos níveis desta marca no núcleo lesado em comparação com os núcleos não lesados na mesma célula, sugerindo uma diminuição na quantidade de genes transcripcionalmente ativos. Contudo, e como referido anteriormente, trata-se de um resultados preliminar que pretendemos comprovar brevemente. Adicionalmente, pretendemos testar outras marcas epigenéticas descritas como características de cromatina ativa e inativa de modo a comprovar a nossa hipótese.

Todos os resultados obtidos demonstram que ambos os mioblastos e os miotubos são capazes de corretamente sinalizar e ativar a DRR. A ausência de *foci* de RAD51 em resposta às lesões induzidas permite excluir a hipótese de que os miotubos são capazes de utilizar a via canónica de HR para reparar DSB, enquanto a sua presença nos mioblastos indica o contrário. Os ensaios realizados para avaliar as transições no ciclo celular revelaram que os miotubos

não sofrem alteração no ciclo celular e não replicam o seu DNA como mecanismo para reparar eficientemente as lesões.

A ativação de p21 em resposta a lesões no DNA sugere a ativação do controlo do ciclo celular, contudo o seu papel nos miotubos tem de ser clarificado. As modificações na cromatina observadas após lesão no DNA com irradiação laser UV-A sugerem uma inativação metabólica do DNA lesado como parte de um mecanismo para inativar excessivas lesões num único núcleo sem comprometer a sobrevivência celular. Esta hipótese será futuramente testada.

Palavras- chave: Resposta a danos no DNA, quebras na dupla cadeia de DNA, controlo do ciclo celular, células de músculo esquelético.

Abstract

The DNA damage response and the effective pathways used to repair DNA lesions have been largely studied in proliferating cells. However, less is known on how differentiated cells, like myotubes, can accurately and efficiently repair severe DNA lesions like double-strand breaks (DSB) and what role can cell cycle transitions have in this field. Our results show that differentiated myotubes cannot repair DNA DSB by the canonical homologous recombination (HR) pathway. In fact, we did not find any evidence of cell cycle re-entry upon inducing DNA lesions. Moreover, we found that, upon DNA damage of one single nucleus, myotubes do not commit to apoptosis. Instead, we observed a rearrangement of the architectural features of chromatin, which is an important aspect of the DNA damage signalling and repair. Namely, we found that DNA damage triggers a chromatin condensation state that is suggestive of a global transcriptional shut-off. Our data suggest that selective inactivation of one single nucleus with damaged DNA is part of the DNA repair toolbox of multinucleated cells. With this new tool, myotubes could prevent the transcription of aberrant genes while avoiding apoptosis to maintain cell viability.

Key-words: DNA damage response, double-stranded breaks, cell cycle checkpoint, skeletal muscle cells.

Introduction

Chromatin, DNA Integrity and DNA Damage Response

The integrity of genetic information is of utmost importance for cell viability. Deoxyribonucleic acid (DNA) is the molecule that stores the genetic information in the cell and consists in two polynucleotide chains held together by hydrogen bound between the base portions of the nucleotides.

Two meters human DNA molecule interacting with histones – i.e. chromatin – condense and pack into the nuclei of each cell (Peterson & Laniel, 2004). This individual isolated organelle surrounded by a nuclear envelope regulates gene expression, replication, and cellular growth and protects the DNA of reactive agents present in the cytoplasm. However, this envelope is permeable to small molecules that can cause alterations in DNA. Such alterations in DNA can result in mutations and lead to disease. Every day our cells are exposed to several sources of damage, some of them are endogenous and, in certain levels, are related with normal metabolism, like reactive oxygen species. Others are exogenous and derived from exposure to certain environmental mutagenic factors (Ciccia & Elledge, 2010).

It is estimated that in each cell spontaneous DNA alterations occur more than 100000 times per day and approximately 50 of them are double-strand breaks (DSB) (J. H. Hoeijmakers, 2009). These spontaneous lesions can arise during DNA replication by defects in the incorporation of deoxyribonucleotide triphosphate (dNTPs), interconversion between DNA bases caused by deamination, loss of DNA bases following DNA depurination and modification of DNA bases by alkylation (Lindahl & Barnes, 2000).

Examples of environmental sources of DNA damage are ultra-violet (UV) light and ionizing radiation (IR). Chemical agents present in medical treatments for cancer patients, industrialized food and cigarettes also cause a variety of lesions in DNA. UV light is one of the most incisive source of damage: in strong sunlight about 100000 of lesions per exposed cell per hour can be caused by

residual UV-A and UV-B (J. H. Hoeijmakers, 2009; Jackson & Bartek, 2009). IR from cosmic radiation, medical treatments and medical diagnostic can induce oxidation of DNA bases and, consequently, generate single-strand breaks (SSB) and DSB (Ciccia & Elledge, 2010).

Independently of the origin of the mutagenic factors, they can lead to insertions, deletions, adducts, intra and interstrand crosslink, DNA SSB and DSB. DSBs are the most catastrophic form of DNA damage and their occurrence compromise genomic stability (J. H. Hoeijmakers, 2009). In order to maintain homeostasis, ensure cell viability and survival, cells evolved many different mechanisms to repair different types of damage (Lindahl & Barnes, 2000; Vilenchik & Knudson, 2003).

The damaged DNA has to be recognized and flagged in order to be repaired. Altogether, these tree fundamental components - sensing, signaling and repair – constitute the DNA damage response (DDR) (Soria, Polo, & Almouzni, 2012). Chromatin plays a crucial role in the DDR: as a first step, changes in chromatin structure act like a flag for the recruitment of DNA signaling and repair factors and second, chromatin remodeling is needed for giving access to repair machinery (Deem, Li, & Tyler, 2012). Eventually, chromatin condensation and heterochromatin formation may arise in nuclei with damaged DNA.

Double Strand Break Repair

There are two major cellular mechanisms that can repair DSB: homologous recombination (HR) or Non-Homologous End-Joining (NHEJ). In contrast to HR that requires a homologous and undamaged sequence to repair broken DNA, NHEJ pathway directly ligate broken DNA in an error-prone manner (Lieber, 2008; Symington & Gautier, 2011). To serve as a donor template for HR, homologous sequences could be present anywhere in the genome: sister chromatid (the preferential one to repair DNA in an error-free manner),

homologous chromosome and repetitive regions in the genome (Renkawitz, Lademann, & Jentsch, 2014).

In response to DNA damage, the two first groups of proteins that mediate DDR and are recruited to the break site are the Mre11-Rad50-Nbs1 (MRN) complex (van den Bosch, Bree, & Lowndes, 2003) and the phosphatidylinositol 3-kinase related protein kinase (PIKK) family: ataxia-telangiectasia mutated (ATM), Rad3-related protein kinase (ATR), and DNA dependent protein kinase (DNA-PK) (Harper & Elledge, 2007; van den Bosch et al., 2003).

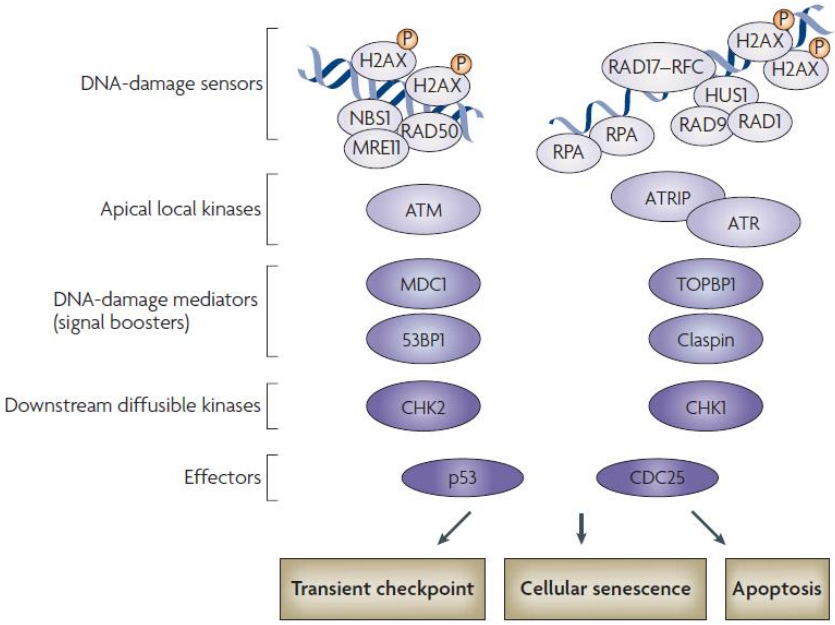

Figure 1. DNA damage response. Adapted from Fabrizio d´Adda di Fagagna Nature reviews cancer

The MRN complex rapidly senses and accumulates at the DNA DSB, recruiting ATM. At the DSB, ATM auto phosphorylates and dissociates from the DNA becoming active (Bakkenist & Kastan, 2003; van den Bosch et al., 2003). This activation will allow ATM to phosphorylate the histone variant H2AX on Ser139 (γH2AX) in a region spreading up to 2 megabases away from the DSB, providing a binding site for MDC1 (Burma, Chen, Murphy, Kurimasa, & Chen, 2014). Moreover ATM itself will recruit repair factors, like breast cancer susceptibility protein 1 (BRCA1) and p53 binding protein 1 (53BP1), that will expand and amplify the DNA damage signaling and determine which pathway will be chosen for repair (Daley & Sung, 2014; Maréchal & Zou, 2013). During the G1 cell cycle phase, 53BP1 prevents the recruitment of BRCA1-partner and

localizer of BRCA2 (PALB2)-breast cancer susceptibility protein 2 (BRCA2) to the DSB by impairing the recruitment of BRCA1, hence suppressing HR (Bunting et al., 2010; Escribano-Díaz et al., 2013).

Checkpoint kinase 1 and 2 (Chk1 and Chk2, respectively) are both phosphorylated in diverse genotoxic conditions by ATR and ATM kinase, respectively (Bartek & Lukas, 2003). Their functions are related with the signalling of the DNA damage from the upstream kinases, providing the recruitment of repair factors and inducing cell cycle arrest.

Non Homologous End Joining Pathway

Most mammalian cells spend the majority of their live time in G1 phase of the cell cycle. Because of that the mechanism that will be preferential adopted will be the NHEJ pathway (Mao, Bozzella, Seluanov, & Gorbunova, 2008). This mechanism will join together the broken ends. So repair of DSB by NHEJ proceeds in three different steps: End-binding, end-processing and ligation.

The first complex binding a DSB is the KU70/80 heterodimer (Davis & Chen, 2013). This abundant KU70/80 complex has high affinity to DNA ends and serves as a scaffold to allow the recruitment of other NHEJ factors and prevent excessive processing of the broken DNA ends. Proteins such as DNA-PKcs, DNA ligase V, X-ray cross complementing protein 4 (XRCC4) and XRCC4-like factor (XLF) bind through the KU70/KU80 complex (Costantini, Woodbine, Andreoli, Jeggo, & Vindigni, 2007; Uematsu et al., 2007). After being recruited by the KU70/80 complex, DNA-PKcs will undergo autophosphorylation, which will activate its kinase activity allowing the phosphorylation and activation of many downstream targets, like Artemis. Artemis access to DNA results in the processing of non-connectable ends. The Artemis:DNA-PKcs complex can act as a 5’ or 3’ endonuclease at DNA overhangs (Lieber, 2008; Meek, Dang, & Lees-Miller, 2008). The XRCC4/DNA ligase IV complex carries out the final end-joining step in NHEJ, resulting in the ligation of the broken ends. This process is

therefore not guided by a DNA template which leads to an error-prone DNA repair (Lieber & Wilson, 2010).

Homologous Recombination Pathway

HR provides an accurate repair of DNA DSB (Takata et al., 1998). The preferential use of sister chromatid to accurately repair the DSB restricts this mechanism to late S and G2 phases of the cell cycle (San Filippo, Sung, & Klein, 2008; Takata et al., 1998). Broken ends are processed to yield 3´ single stranded DNA (ssDNA) tails, in a mechanism named end resection, where 5´-3´nucleolytic degradation of DNA ends takes place. The initial resection is made by the CtBP-interacting protein (CtIP) and the MRN complex and the extensive resection is performed by BLM helicase associated with EXO1 and DNA2 (Nimonkar et al., 2011).

DNA resection will activate a set of protein kinases described before. These proteins are ATR, ATM and DNA-PK which regulate replication protein A (RPA) activity by promoting its phosphorylation. RPA binds to ssDNA tails but is further replaced by RAD51 with the help of BRCA2.

Binding of RAD51 to the single DNA strand promotes the invasion into a double stranded DNA to search for a homologous sequence. Homologous pairing recruits polymerase δ and proliferating cell nuclear antigen (PCNA) to perform the strand extension (Li, Holzschu, & Sugiyama, 2013; Pospiech, Rytkönen, & Syväoja, 2001).

Both NHEJ and HR repair pathways play a role in maintaining chromosomal integrity during the cell cycle (Takata et al., 1998). Defects on HR mechanism are associated with mutagenesis and predispose to cancer which strengthens the importance of these repair pathways for preserving genome integrity (Moynahan & Jasin, 2010).

Cell Cycle Checkpoint and DNA repair

During the DDR, cell cycle checkpoints halt the cell cycle progression providing sufficient time for the cell to repair the DNA molecule (Kastan & Bartek, 2004).

At the core of this highly controlled mechanism are cyclin-dependent kinases (CDK) and cyclins. CDK levels are relatively constant in the cell, but cyclins are extremely variable. The binding of cyclins to CDK activate their catalytic activity, and is responsible for sending signals to responder molecules that move the cell through the cell cycle.

The main pathway through which DNA DSB induce cell cycle arrest is by increasing the levels of p53. The tumour suppressor p53 is a transcription factor that amongst other genes, activates the transcription of p21, which acts as a potent CDK inhibitor (Macleod et al., 1995).

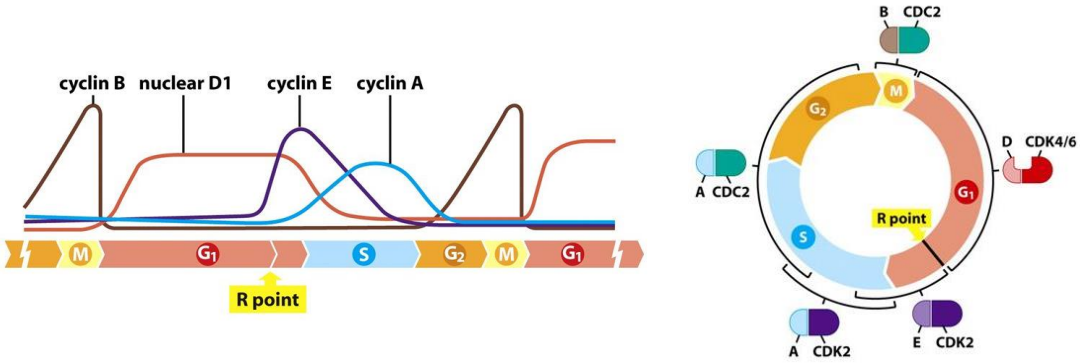

Figure 2. CDK complexes during cell cycle. Adapted from The Biology of Cancer (©Garland Science 2014)

DNA Repair in Post-Mitotic Cells

Dividing cells tend to repair DNA errors more accuratly than non-dividing cells (J. H. J. Hoeijmakers, 2015). In theory, a terminally differentiated cell can not repair a DSB through error-free HR because the sister chromatin is not available (Iyama & Wilson, 2013). Since the lifespan of a terminally differentiated cell (i.e. a non-dividing cell with specific structural, functional, and biochemical properties (Iyama & Wilson, 2013)) could be decades, it is crucial that a competent DNA repair strategy is available to prevent premature aging and disease.

In comparison to dividing cells, little is known about the DDR in post-mitotic cells, such as neurons and skeletal muscle myotubes. These cells undergo cell cycle withdrawal with concomitant downregulation of the major DNA repair pathways. Whether myotubes undergo G0-G1 transitions upon DNA damage has not been investigated but it is known that neurons are able to re-enter the cell cycle for DNA repair (Kruman et al., 2004). In neurons, the attempt to progress through the cell cycle in response to persistent DNA damage does not result in cell division. Instead, it culminates in cell death by p53-dependent checkpoint activation and apoptosis. In multinucleated myotubes, the DNA damage-signalling cascade that leads to p53 activation is defective, suggesting that different mechanisms exist to cope with accumulated DNA damage (Fortini et al., 2012).

Post-Mitotic cells – the notorious example of skeletal muscle myotubes

Skeletal Muscle is a complex tissue that plays a multitude of functions. The most important functions of skeletal muscle are the conversion of chemical energy into mechanical energy in order to generate force and power, the maintenance of posture and the production of movement (Frontera & Ochala, 2015). Skeletal myogenesis – the fusion of myoblasts into multi-nucleated myotubes ending in terminally differentiated muscle myofibers - is a highly ordered process of temporally separable events that begins with myogenin expression in myoblasts, followed by cell cycle arrest, phenotypic differentiation, and finally, cell fusion (Andres & Walsh, 1996). There are two important classes of myogenesis: *de novo* embryonic myogenesis and adult myogenesis.

All skeletal myogenic cells migrate and differentiate in response to different biological signals (Ordahl & Le Douarin, 1992). Muscle progenitors can take two different roads: they can either proliferate or they can exit the cell cycle and commit to fuse into terminally differentiated muscle myofibers. The balance between the proliferation of muscle progenitors and their differentiation gives a harmonious growth balance of skeletal muscle. In adult myogenesis, the maintenance of muscle integrity and homeostasis relies on muscle regeneration upon damage. For that, the recruitment of muscle undifferentiated cells with myogenic differentiation capacity – mostly satellite cells - is needed (Bentzinger et al., 2012). When activated, these mitotically quiescent cells divide asymmetrically for self-maintenance and for muscle progenitor's pool (Cornelison & Wold, 1997; Kuang, Gillespie, & Rudnicki, 2008).

The entire myogenic process is tightly controlled by a broad range of genes that work like key regulators of muscle progenitor cell specification and differentiation. The expression of several myogenic regulators factors (MRFs) establishes a commitment to myogenic program. One important protein expressed by induction of MRFs is myosin heavy chain (MHC). MHC is expressed in skeletal muscle myotubes and fibres and is currently used to control and follow skeletal differentiation process (Delbono, 2010; Molkentin & Olson, 1996).

Aim

Given the scarce knowledge of the DDR in terminally differentiated cells, we aim at elucidating the molecular mechanisms of DNA repair in these cells, using skeletal myotubes as a model system. We will test the hypothesis that terminally differentiated myotubes transiently re-enter the cell cycle in order to repair damaged DNA. Moreover, since these are multinucleated cells, we will inspect if the DDR to DNA damage in all nuclei of a single myotube involves the same processes as the DDR to DNA damage in a single nucleus. We reason that while persistent DNA damage on several nuclei triggers apoptosis, DNA damage on a single nucleus does not elicit myotubes death, but rather a DDR pathway that maintains cellular viability.

Material and Methods

Myoblasts Cell Culture and Differentiation induction into myotubes

KM155 cell line (Human Skeletal Myoblasts) was kindly provided by Maria do Carmo Fonseca Lab that has received them from Vincent Mouly from the Institut de Myologie UPMC Université Paris 6 France (Mamchaoui etc al 2011).

The undifferentiated myoblasts were grown in Skeletal Muscle Cell Media (PromoCell) supplemented with a mix containing Fetal Calf Serum (0,05 ml/ml), Fetuin (50 µg/ml), Epidermal Growth Factor (10 ng/ml), Basic Fibroblast Growth Factor (1 ng/ml), Insulin (10 µg/ml) and Dexamethasone (0,4 µg/ml). To make myoblasts differentiate into myotubes the medium was changed to differentiation medium (serum starved). The differentiation medium contains equal quantity of Iscove's Modified Dulbecco's Medium (IMDM) and Ham's F-10 Nutrient Mixture (F-10) (both from Gibco) supplemented with 1% ITS (I1884 Sigma Aldrich). Half of the differentiation medium was changed every other day until day 5.

When cells were seeded in glass coverslips a previous coating with 0,2% porcine gelatine (G1393, Sigma Aldrich) was needed. Both cells were maintained at 37°C in a humidified atmosphere containing 5% CO2.

Index Fusin Calculation

In order to determine and quantify differentiation efficiency, muscle cell nuclei within myotubes and nuclei within unfused cells were counted. The fusion index was calculated as the percentage of nuclei belonging to cells possessing three or more nuclei on total of nuclei counted. Approximately 300 to 500 cells were counted each time.

Drug treatments

To induce DNA damage, cells were treated with neocarzinostatin (NCS) at 250 ng/ml (N9162, Sigma Aldrich) for 30 min, washed twice and harvested or fixed immediately after the treatment and at the indicated time-points (see Results section). In live cell microscopy experiments using FUCCI system NCS was added at 500ng/mL, washed twice and allowed to recover in fresh medium.

Protein extracts and Western Blot

Whole cell protein extracts were prepared by cell lysis with SDS-PAGE buffer (80 mM Tris-HCL pH 6.8, 16% glicerol, 4.5% sodium dodecyl sulfate (SDS), 450 mM dithiothreitol (DTT), 0.01% bromophenol blue) with 100U benzonase (E1014-25KU, Sigma Aldrich) and 50 µM $MgCl_2$. The lysates were incubated for 20 minutes and boiled for 6 min at 100°C. Equal amounts of protein extracts and a protein maker (MB09002, NZYTech) were loaded in 8% or 12% acrylamide gel according to molecular weight of the proteins). The gels were resolved in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (IB301031, Life Technologies).

Resulting membranes were blocked with milk solution (5% m/v in PBS 1X - 0,05% tween 20) for 1 hour at room temperature. After this, membranes were incubated overnight at 4ºC with the primary antibodies and gentle shaking, followed by washes with PBS1X-0,05% Tween and incubation for 1 hour at room temperature with the appropriate horseradish peroxidase (HRP)–coupled secondary antibody. Detection reaction was performed with chemiluminescence substrates (RPN 2134, Amersham and 34096, Thermo Fisher Scientific).

Immunoblotting was performed with antibodies against the following proteins: 53BP1-P (phosphoSer1778; No.2675, Cell Signaling, Danvers, MA), ATM-P (phosphoS1981; No.200-301-400s, Rockland, Gilbertsville, PA), α-Tubulin (T5168; Sigma Aldrich); γH2AX (phosphoSer139; 05-636, Millipore); total p53 (sc-263; Santa Cruz); p21 (sc-397; Santa Cruz); MHC (MF20, Developmental Studies Hybridoma Bank).

yH2AX and RAD51 Immunofluorescence

KM155 cells grown on coverslips were fixed with 3.7% paraformaldehyde for 10 min at room temperature. The myoblasts and myotubes were then permeabilized with 0,5% or 1% Triton X-100/PBS, respectively, for 10 min. Incubation step with primary antibodies against γH2AX (phosphoSer139; 05–636, Millipore) and RAD51 (ab213; Abcam) for 1h at 37°C was followed by incubation with fluorochrome-conjugated antibodies Alexa Fluor 488 anti-mouse (A-21202, Life-Technologies) and Cy3 anti-mouse (115-166-003, Jackson Immunoresearch). All the washing steps were done with PBS containing 0.05% (vol/vol) Tween 20. The samples were mounted in Vectashield mouting medium (H-1000; Vector Laboratories, Burlingame, CA) with 4'-6-diamidino-2-phenylindole (Dapi) (09542; Sigma Aldrich) to stain the DNA. A Zeiss LSM 710 confocal point-scanning microscope (Carl Zeiss) was used to visualize the cells with a 40x/1.4 oil immersion.

Edu Flow Cytometry Assay

KM155 myoblasts and myotubes cells grown on P35 were treated with NCS at 250 ng/ml for 30 minutes and allowed to recover. During this recovery time, cells were incubated with 5-ethynyl-2´-deoxyuridine (EdU) (10μM) at different time point intervals: 0-2h, 2-4h, 4-6h, 6-8h. Cells without treatment were incubated with EdU for 2h in order to measure the basal levels of EdU incorporation.

Cells were washed with cold PBS 1X, scraped out, transferred to an Eppendorf and centrifuged at 4000 rpm for 5 min at 4ºC. The process of fixation, permeabilization and click-iT reaction was done according to the manufacturer's protocol (C-10425 Click-iT EdU Flow Cytometry Assay Kit protocol, Thermo Fisher Scientific). Cells were immediately analysed with BD Accuri C6.

Treatment with ATM Inhibitor

Myotubes were incubated with 5μM ATM inhibitor KU-55933 (abcam) for 1h. The inhibitors were kept in the cell culture medium during the following treatment with NCS for 30 minutes. After NCS treatment, cells were washed two times and allowed to recover, always in the presence of the inhibitors. As a control condition, cells were maintain without adding inhibitor. Also for each condition we maintained cells not treated with NCS. After this, cells were lysed and the extracts collected at different time points of recovery: 0, 1, 2, 6 and 18 hours.

Transfections and live imaging visualization

Transfection of the cells was performed with Lipofectamine® 3000 transfection reagent (L3000015, Invitrogen). Two solutions were prepared, one containing 125μL of Opti-MEM Reduced Serum Medium (Opti-MEM) (31985070, Gibco), plasmid DNA, P3000 reagent (2μL of P3000 per 1μg of DNA) and other containing the same amount of Opti-MEM and Lipofectamine 3000 reagent (1,5μL of lipofectamine per each μg of DNA). Both solutions were incubated for 5 minutes separately and after this time, the solution containing the DNA was added to lipofectamine solution. After 20 minutes the mixture was added to the medium. The plasmids used to transfect cells were: pUBC-H2B-GFP, mKO2-hCdt1(30/120) and mAG-hGeminin(1/110).

A 3i Marianas SDC spinning disk confocal microscope (Intelligent Imaging Innovations, Inc.) was used to visualize the cells transfected with Fucci system. Multiple positions were selected before the time series imaging protocol was initiated, cells were then imaged for 64 time points with 15 min intervals covering a 16 h total observation time. At every position a z-stack of 4 optical planes with 0.63 μm step size, respecting Nyquist sampling, was acquired. Imaging was performed in 2 fluorescence channels sequentially at every image z-plane using filter sets for GFP (510-540 nm bandpass) and red fluorescent proteins (580-653 nm band pass).

Zeiss LSM 880 confocal point-scanning microscope (Carl Zeiss) was used to visualize myotubes transfected with pUBC-H2B-GFP as a live cell fluorescent chromatin label to visualize the number and position of nuclei, as well as document the large scale nuclear chromatin architecture. Following the UV-A induced DNA damage, cells were kept for 24h under standard culturing conditions and imaged again to monitor and analyse the chromatin architecture 24 hours after damage. During image acquisition, the cells were kept at 37°C with 5% CO2 and 100% humidity.

The pUBC-H2B-GFP was previously generated in our lab by ligating a BglII + BamHI fragment from pH2B-mRFP containing the H2B open reading frame including a nuclear localization sequence (described in Martin et al. 2010 FASEB J., Apr; 24(4): 1066–1072) into the BglII site of of a pUBC-GFP-C1 vector. The empty pUBC-GFP-C1 vector was made by inserting the human ubiquitin C promoter sequence from phage-ubc-nls-ha-tdMCP-gfp (Addgene plasmid # 40649, a gift from Robert H. Singer) into pEGFP-C1 (Clontech, Catalog #6084-1) to replace the CMV promoter.

Measurement of H3K36me3 relative intensities

To measure H3K36me3 intensity levels acquired images were analysed using ImageJ program. Background was subtracted and was measure the stain of H3K36me3 that match with DNA staining (DAPI) for each nucleus and each stack. Normalize to the highest total value.

Results and Discussion

From Myoblasts to Myotubes: Serum-free medium coupled to rich confluence enhances myotube formation

Differentiation of postmitotic multinucleated myotubes from myoblasts can be performed *in vitro* under serum starvation (Lawson & Purslow, 2000; Yaffe & Saxel, 1977). We started this project by optimizing myogenic differentiation in order to obtain postmitotic skeletal muscle myotubes, progenitors of myofibers. Myoblasts cultured in medium supplemented with serum and additional growth factors proliferate and do not differentiate. In contrast, high cell confluence together with low serum content is sufficient to activate myogenic differentiation and cell fusion (figure 3A).

A network of transcription factors that control skeletal muscle development is activated resulting in substantial changes in the expression of muscle specific genes, like myosin heavy chain (MHC) (Braun & Gautel, 2011). In order to induce the myogenic process, we cultured confluent myoblasts under serum starvation during 5 days. MHC levels measured by western blot during differentiation, confirmed the efficiency of the process (figure 3B). The morphological evaluation of the cells further suggests that myotubes are efficiently differentiated after 5 days (figure 3A and B). However, unfused progenitors and fusion cells halted in primary stages of differentiation were also observed in the cultures.

Therefore, in order to access the efficiency of the myogenic process, we measured the index fusion of the resulting myotubes. The index fusion is calculated by the percentage of nuclei belonging to cells possessing three or more nuclei over the total number of nuclei counted. The results revealed that we are able to obtain around 65 to 70% of multinucleated myotubes (figure 3C).

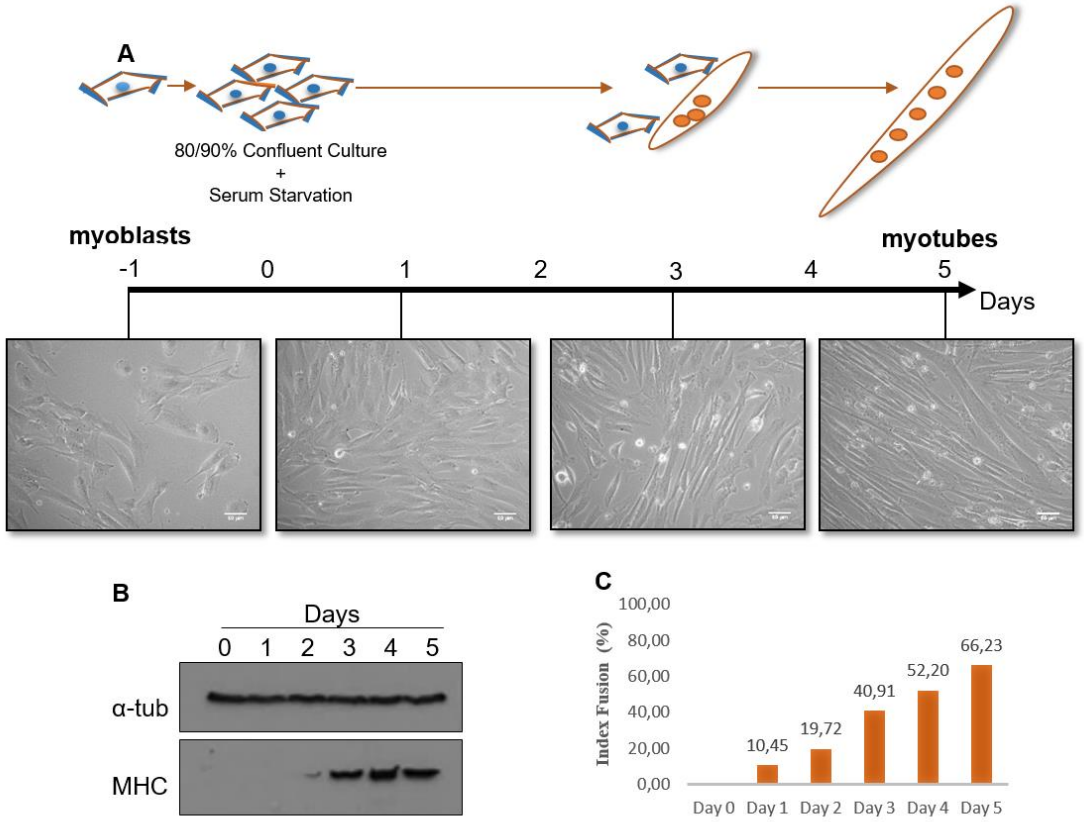

Figure 3. During cell differentiation, cells become multinucleated and increase MHC expression. (A) Schematic representation of the differentiation process and representative images for day -1 (myoblasts), day 1, day 3 and day5 (myotubes). (B) Total protein extracts of KM155 cells undergoing differentiation were analysed by Western blot, with antibodies directed against MHC (223kDa) and α-tubulin (55kDa), as indicated on the left, following different time points after inducing damage with NCS. NCS treatment is depicted as (-) for no treatment, (´) for minutes and (h) for hours after damage induction. We used α-tubulin as loading control. The blot represents the result of one experiment from two independent experiments performed with similar results. (C) Index fusion calculation was done for every single day, from day 0 until day 5 of myogenic differentiation process.

The DDR in myotubes and myoblasts

When the cell faces a DNA DSB a decision has to be made in order to avoid genomic instability and ensure cell integrity: which pathway should repair the damaged DNA?

As previously described, there are two major mechanisms to repair DSB: HR and NHEJ. In order to inspect if both pathways are available in skeletal muscle myotubes, we induced DNA damage using NCS, an antibiotic that intercalates in the minor groove of DNA and produces DSB by complex radical mechanisms, mimicking the effect of ionizing radiation (Obe, Johannes, & Ritter, 2010). After NCS treatment, myoblasts and myotubes were chased in fresh media during different time points. Myosin heavy chain (MHC) was inspected to control for the differentiation process and, indeed, it was only expressed in differentiated myotubes and not in myoblasts (figure 4). The western blot analysis of the levels of several DNA repair factors allowed us to evaluate the dynamics of the DDR (figure 4). The phosphorylation of H2AX was observed both in myoblasts and myotubes (figure 4). We observed a complete recovery of γH2AX to basal levels in myoblast and myotubes, after 2h and 6h of the induced damage, respectively. In comparison to myoblasts, myotubes have more γH2AX in the absence of NCS, indicative of higher endogenous DNA damage events. In fact, it is expected that non-proliferative cells do accumulate more DNA damage than proliferating cells, given the decreased frequency or even absence of HR. In addition to γH2AX, phosphorylated ATM (ATM-P) and 53BP1 (53BP1-P) were also observed upon NCS treatment of both myoblasts and myotubes (figure 4). However, phosphorylation of these proteins remained at higher levels and for a longer time period in myotubes than in myoblasts (figure 4). These results allow us to conclude that the DDR is properly activated in both myoblasts and myotubes although with seemingly different dynamics.

As for the CDK inhibitor p21, we detected its activation in both myoblasts and myotubes approximately 4 hours after DNA damage with NCS (figure 4). This finding is suggestive of activation of cell cycle checkpoints in response to DSB. However, while in proliferating myoblasts, p21 activation can be related with the blocking of the cell cycle progression to allow the DNA repair, in non-dividing

myotubes cell cycle checkpoint activation may not be the reason why p21 expression increases. Instead, p21 activation in myotubes may be indicative of apoptosis, another function on which this protein has already been implicated (Piccolo & Crispi, 2012). This particular aspect deserves further investigation.

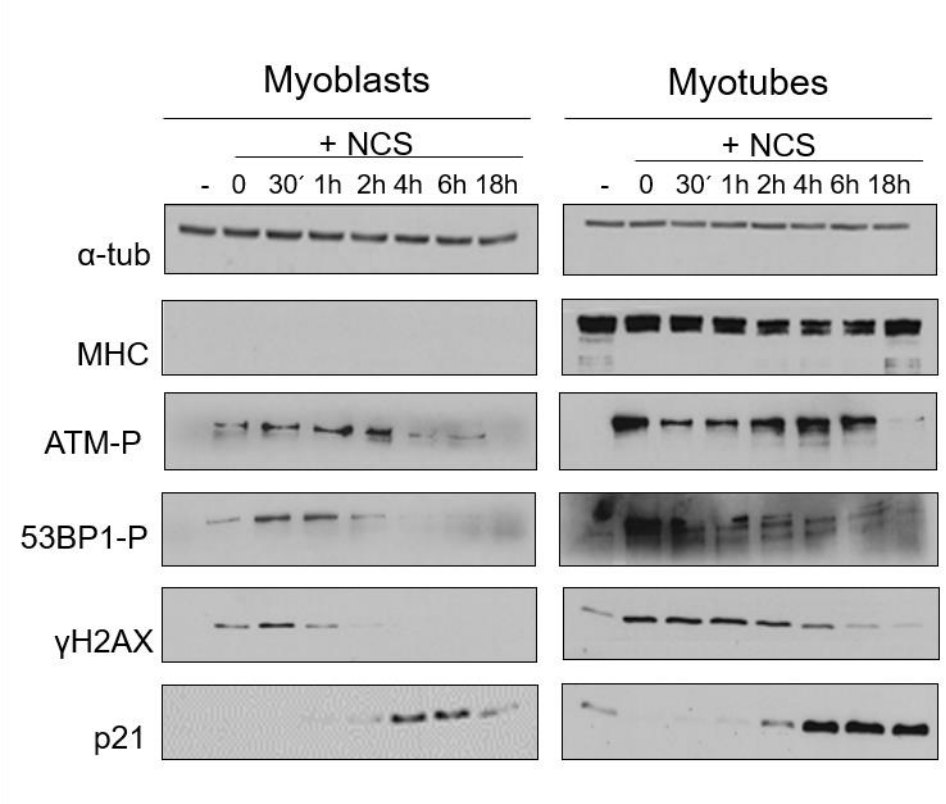

Figure 4. Myoblasts and Myotubes exhibit different phosphorylation dynamics of DDR and cell cycle related factors. Total protein extracts of KM155 myoblasts and myotubes were analysed by Western blot with antibodies directed against MHC (223kDa), ATM-P (370kDa), 53BP1-P (450kDa), γH2AX (17kDa), p21 (21kDa) and α-tubulin (55kDa) as indicated on the left. Following different time points after inducing the DNA damage with NCS. NCS treatment is depict as (-) for no treatment, (´) for minutes and (h) four hours after DNA damage induction. α-tubulin as loading control. The figure represents the result of one experiment from three independent experiments performed with similar results.

The ATM signalling activates p21 in myotubes

Since ATR is not expressed in myotubes (Lukas et al., 2001) we sought to investigate if phosphorylation of H2AX and p53/p21 activation in these cells is ATM-dependent. For that we treated cells with the specific ATM inhibitor, KU-55933 and followed the kinetics of γH2AX and p53/p21 in response to NCS. In response to DNA damage, ATM activation proceeds through an auto-phosphorylation event (Bakkenist & Kastan, 2003). Hence, in agreement with an efficient ATM inhibition, we did not detect any ATM phosphorylation (ATM-P), upon DNA damage in cells treated with the inhibitor (figure 5). Moreover, the levels of γH2AX, p53 and p21 upon DNA damage were severely reduced in myotubes treated with the ATM inhibitor (Figure 5). These data suggest that the DDR signalling in myotubes is dependent of ATM.

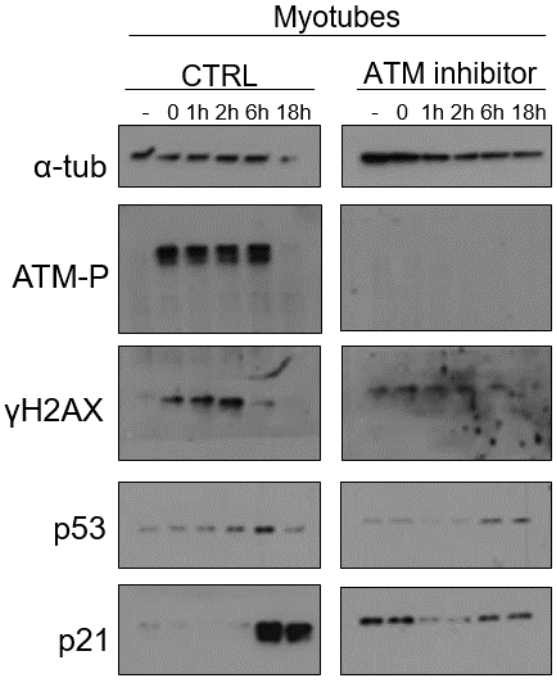

Figure 5. ATM signaling activates p21 in myotubes. Total protein extracts of KM155 myotubes previously incubated with ATM inhibitor (KU-55933) were analysed by Western blot with antibodies directed against ATM-P (370kDa), γH2AX (17kDa), p53 (53kDa) and p21 (21kDa) as indicated on the left. Following different time points after inducing the DNA damage with NCS. NCS treatment is depict as (-) for no treatment, (0) for no recovery and (h) four hours after DNA damage induction. α-tubulin (55kDa) as loading control. The figure represents the result of one experiment from two independent experiments performed with similar results

Myotubes do not repair DSB through HR

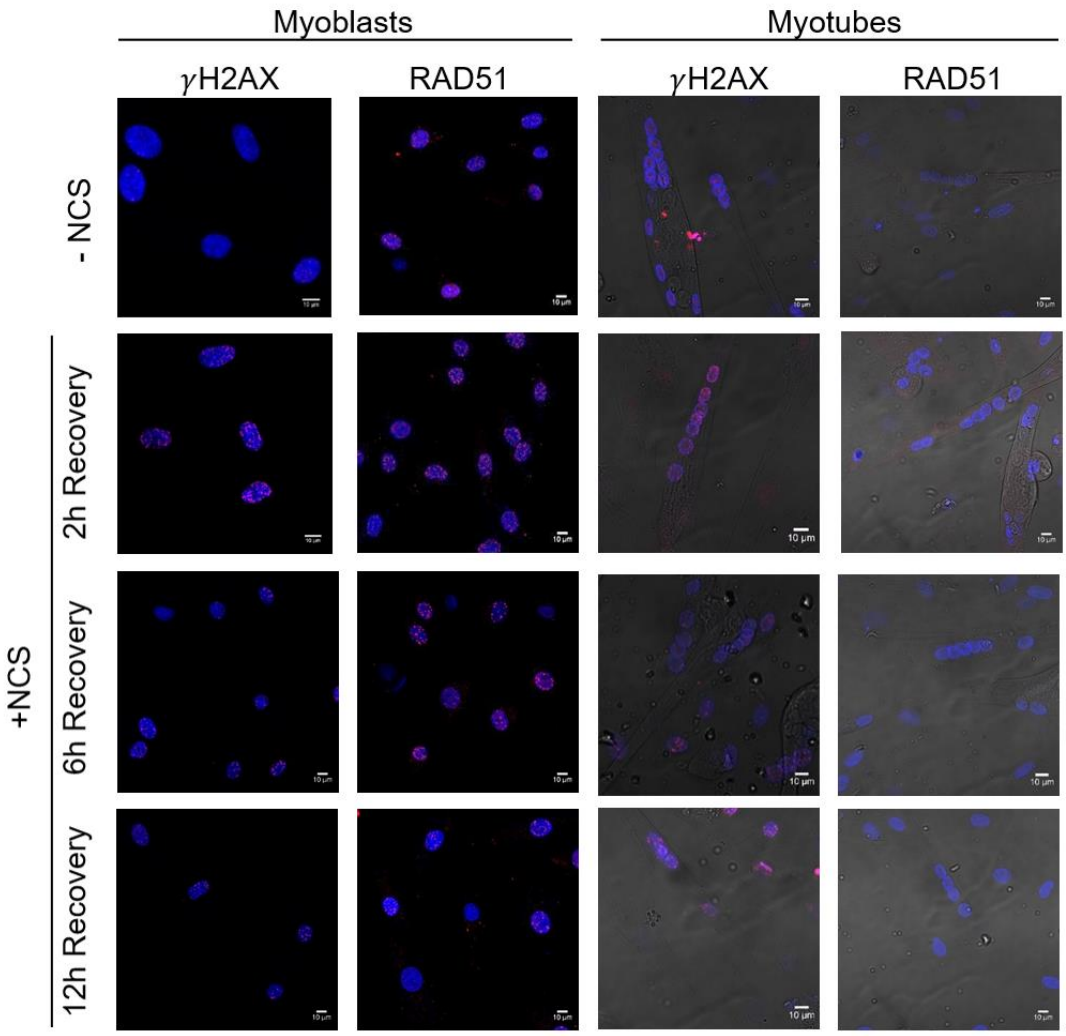

Figure 6. Myoblasts follow canonical homologous recombination pathway to repair DSB but myotubes do not. KM155 myoblasts and myotubes were immunostained with antibodies directed against γH2AX and RAD51, separately, following different time points after inducing DNA damage with NCS. For nuclear staining cells were labelled with DAPI. The figure represents the result of one representative experiment from three independent experiments performed with similar results.

Since ATR is a central component of the HR signalling pathway and taking into account that terminally differentiated myotubes do not replicate their DNA (sister chromatids are the preferred templates for HR), we next sought to investigate if HR was available to repair DSB in myotubes. To assess HR, we measured RAD51 foci formation by immunofluorescence in myoblasts and myotubes challenged with NCS. RAD51 filaments formed on the DNA single-stranded 3′ overhangs are necessary to promote the search for homologous DNA

sequences, thus serving as a reliable readout of HR (Wyman and Kanaar, 2006). In myoblasts RAD51 foci were detected 2 hr and 6 hr after NCS treatment (figure 6). In contrast, RAD51 foci were never detected in myotubes during the entire duration of the experiment (figure 6). Since RAD51 recruitment to DSB is necessary for HR, we conclude that myotubes do not repair DSB through the HR pathway.

Myotubes, unlike post-mitotic neurons, do not enter S-phase during the DDR

Myotubes can re-enter the cell cycle in the absence of retinoblastoma protein (Rb) (Pajcini, Corbel, Sage, Pomerantz, & Blau, 2010). Moreover, post-mitotic cells, like neurons, are able to re-enter the cell cycle as a consequence of DNA damage (Kruman et al., 2004). However, this cell cycle re-entry does not culminate in effective cell division leading only to apoptosis (Kruman et al., 2004). Herein, we aimed at testing if, in addition to neurons, terminally differentiated myotubes undergo cell cycle re-entry during the DDR in order to activate pro-apoptotic programs. To this end, we first used the Fluorescence Ubiquitination Cell Cycle Indicator (FUCCI) system to inspect G1-to-S phase transitions (Sakaue-Sawano et al., 2008) (figure 7 A). In this assay, cells in either G1 or S/G2/M phase are revealed by the selective presence of CDT1-RFP or GEMININ-RFP, respectively. In myoblast cultures, we detected cells in G1 (red nuclei – CDT1-RFP), S/G2/M phase (green nuclei - GEMININ-GFP) and cells preparing to enter S phase (yellow nuclei resulting from the overlap of the GFP and RFP fluorescence signals) (figure 7B). In myotubes, GEMININ-GFP was never detected throughout the entire chase period after DNA damage (figure 7), while the levels of CDT1-RFP were kept constant during the experiment (figure 7C). These results suggest that myotubes do not enter S phase during the DDR.

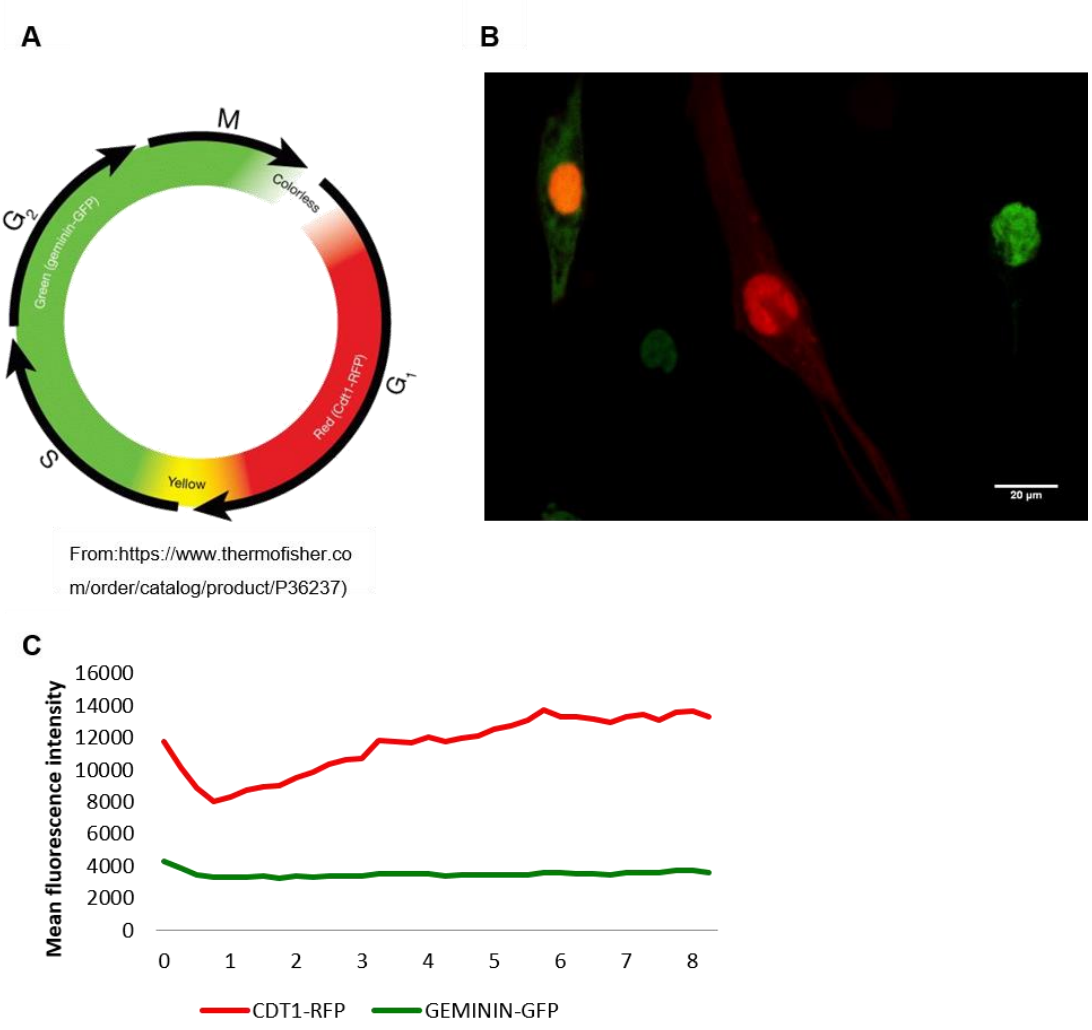

Figure 7. Cell cycle analysis using FUCCI system of myoblasts and myotubes upon damage. (A) Schematic representation of the Fluorescence Ubiquitination Cell Cycle Indicator (FUCCI) system.(B) Proof of concept of FUCCI system in myoblasts. (C) Myotubes previously transfected with the FUCCI system plasmids (Geminin and CDT1, for S/G2/M and G1 cell cycle phase, respectively) were treated with NCS at 500 ng/ml for 30 minutes. Red and green lines represent the fluorescence intensity of mho2 and geminin, respectively. The fluorescence intensity of both plasmids was measured for 8 hours to identify variations in the cell cycle phase upon DNA damage. The graph represents the result of one representative experiment.

To further investigate if myotubes re-enter the cell cycle and proceed into S phase during the DDR, we measured EdU incorporation by flow cytometry (figure 8). In myoblasts, the percentage of myoblasts that incorporated EdU (i.e. that replicate their DNA) decreased during the DDR. In contrast, in myotubes the percentage of cells with EdU incorporation was only marginal and did not change at any of the different time points (figure 8). Altogether these results show that myotubes do not undergo any cell cycle transitions during the DDR. This finding

further strengthens the need for future studies aimed to inspect the purpose of p21 activation (notorious for the role in cell cycle checkpoint activation) in differentiated myotubes.

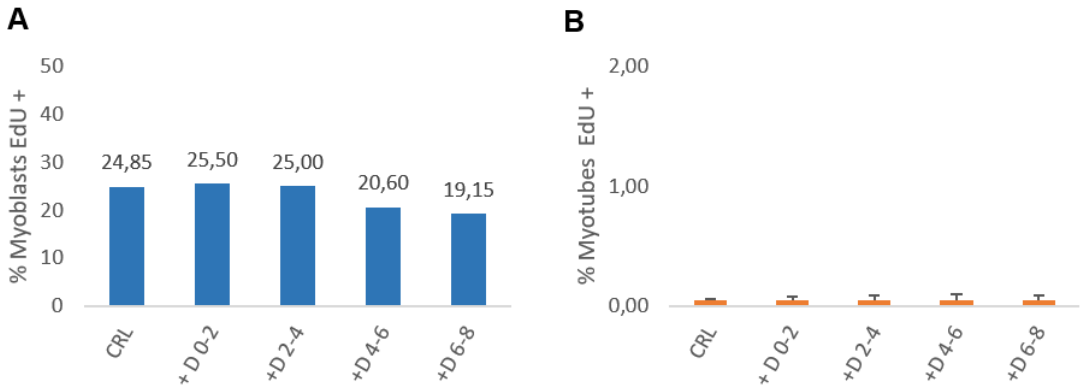

Figure 8. Direct measurement of DNA synthesis in myoblast and myotubes upon DNA damage. FACS quantification of incorporated EdU in myoblasts and myotubes. Cells were treated with NCS and allowed to recover at different time points. Then, to directly measure active DNA synthesis or S-phase synthesis of the cell cycle, cells were incubated with EdU at different time point intervals (indicated in the graphs). (C) and (D) represent respectively, median of two experiments and median for four experiments.

Does DNA damage induce local heterochromatization and transcriptional silencing?

Nucleosome packing and chromatin architecture surrounding the DSB limit the accessibility of the DNA-damage response factors to access and repair DSBs. For example, the increase in acetylation of histone H4 at DSBs promotes an 'open' chromatin conformation allowing for the recruitment of DNA repair machinery (Kruhlak et al., 2006). On the contrary, chromatin condensation has also been described to favour the DDR (Burgess et al. 2014). However, an initial increase in chromatin accessibility followed by condensation is critical to target and signal DNA lesions (Burgess, Burman, Kruhlak, & Misteli, 2014), suggesting that dynamic changes in chromatin architecture take place during the DDR.

To inspect the chromatin dynamics during DNA repair in myotubes, we induced DNA damage specifically in one nucleus of a multinucleated myotube

using UV-A laser irradiation. Chromatin was visualized in cells by the ectopic expression of histone H2B tagged with GFP. We observed that the nucleus with UV-A induced DNA damage exhibited large areas of highly condensed chromatin mostly concentrated in the nuclear periphery when compared with the remaining undamaged nuclei (Figure 9A). The global changes in chromatin architecture observed upon DNA damage suggest a large-scale chromatin condensation mechanism.

To further investigate if chromatin condensation is accompanied by general transcription inhibition in the damaged nucleus, we inspected the effect of UV-A DNA damage on H3K36me3 levels. H3K36me3 was used as a proxy for active transcription(de Almeida et al., 2011; Zhou, Goren, & Bernstein, 2011). Following DNA damage, we observed a 29% decrease in the H3K36me3 levels, quantified from immunofluorescence images (Figure 9B and 9C). We shall nevertheless repeat this experiment including additional histone marks (such as histone H3 lysine 9 acetylation (H3K9ac) or histone H3 lysine 9 trimethylation (H3K9me3)) in order to carefully characterize chromatin modifications upon DNA damage in myotubes and to conclude the effects on transcription. Notwithstanding, these data allow us to propose a model whereby, following DNA damage in a single nucleus of a multinucleated myotube, global chromatin condensation and transcription repression takes place. This would allow the cell to cope with unrepairable levels of DNA damage in one of its nucleus without having to compromise cell viability by undergoing apoptosis. In fact, terminally differentiated myotubes are more resistant to apoptosis than myoblasts (Xiao, Ferry, & Dupont-Versteegden, 2011). Selective inactivation of a single nucleus can illustrate a potential new mechanism that aims to prevent the expression of mutated or damaged genes while maintaining cell survival.

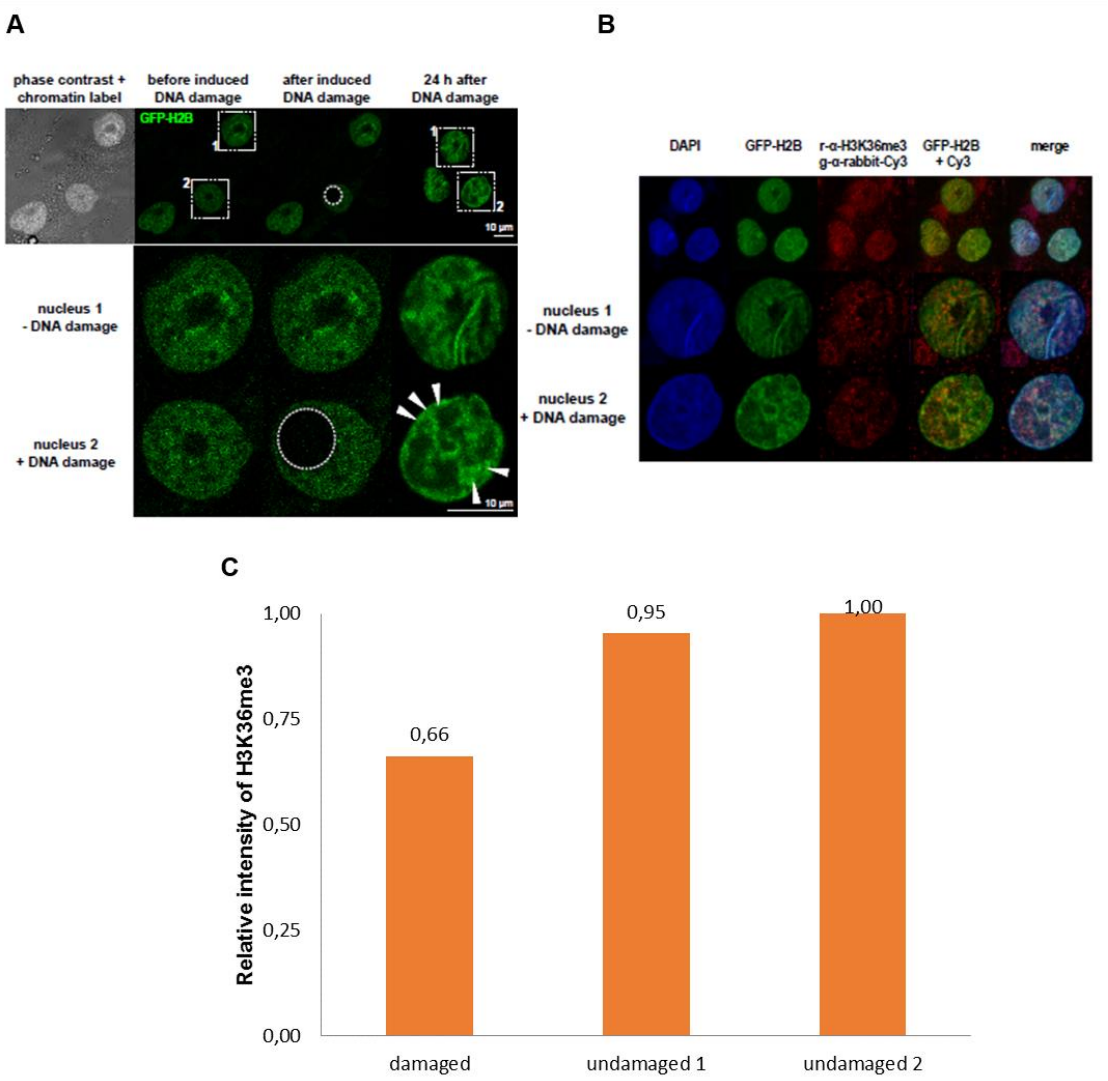

Figure 9. Local condensation of chromatin after UV-A induced DNA damage. (A) The upper row shows a myotube and the phase contrast image with superimposed chromatin (histone H2B fused to GFP) highlighting the location of three nuclei within one myotube. The nuclei were imaged before and directly after inducing DNA damage in one nucleus (2) using a 405 nm laser pulse in the region marked by the dotted circle under a confocal microscope. (B) The cell was revisited after 24 h to monitor the DNA damage induced changes in chromatin architecture in the damaged nucleus (2) compared to undamaged neighbor nuclei (1). The magnified images show one undamaged nucleus (nucleus 1) and the DNA damaged nucleus (2) for direct comparison of the chromatin before and after DNA damage. (C) Quantification of H3K36me3 levels in damaged and undamaged nuclei. The figure represents the result of one experiment.

Conclusions and Future Perspectives

Our results demonstrate that both myoblasts and multinucleated myotubes have proficient DDR pathways with ATM playing a central role on both. However, myoblasts seems to favour canonical HR pathway as visualised by RAD51 nuclear foci in damaged myoblasts, a pathway that is not employed by myotubes, most likely due to their inability to undergo G1-S transitions and replicate their DNA. In agreement, we found that myotubes remain in a quiescent state even after augmented levels of DNA damage. Nevertheless, we observed an activation of p21 during the DDR in myotubes, challenging its major role as a driver of cell cycle arrest. Instead, we reason that p21 may take part in a molecular pathway that culminates in apoptosis of myotubes bearing non-reparable DNA damage. This model is merely speculative and demands further investigation.

Upon DNA damage on a single nucleus of a multinucleated cell we observed chromatin condensation and an overall reduction of H3K36me3 levels, which together are suggestive of a selective inactivation of a single nucleus. This could illustrate a potential new DDR outcome that aims at inactivate nuclei with excessive genomic instability in order to prevent transcription of aberrant (i.e. mutant) genes in myotubes. At the same time, it would preserve cell viability by driving the cell into apoptosis as a last resource when DNA damage is present at all, or most, nuclei. Again additional work is needed to confirm this view.

Altogether, the data presented in this thesis offers new additional insights into the DDR of terminally differentiated multinucleated skeletal muscle cells. By disclosing some specificities of the DDR in myotubes, our work as the potential to pave the way for additional research that may be valuable for our understanding of muscle disorders and age-related conditions, such as sarcopenia.

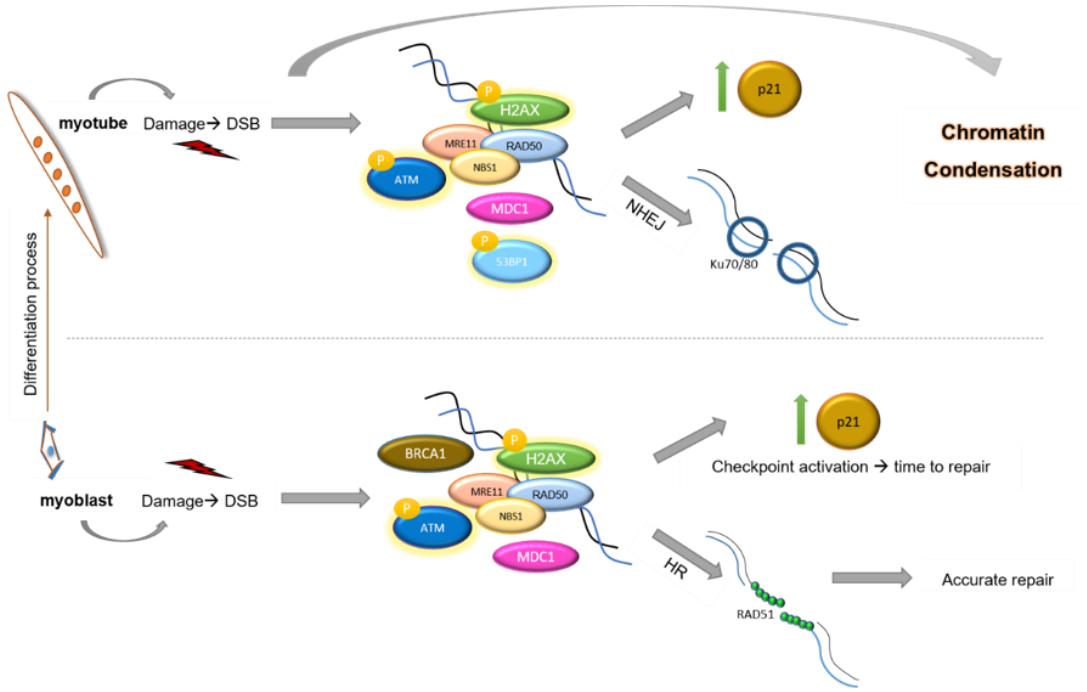

Figure 10. Proposed model for the DDR in myoblasts and myotubes. Undifferentiated myoblasts and differentiated myotubes repair DSB via HR and NHEJ pathways, respectively. In myoblasts, a typical DSB can be repaired through HR starting with the recruitment of BRCA1, followed by end resection and RPA coating of the ssDNA. Consequently, RPA phosphorylated will be replaced by RAD51 promoting strand invasion and repair by an homologous template sequence. In myotubes, DSB repair can only proceed through the NHEJ pathway through were 53BP1 plays important roles.. NHEJ factors like Ku70/Ku80 complex bind to broken ends and recruit other factors culminating in ligation of broken ends. In a single damaged nucleus, chromatin condensation is accompanied by a loss of chromatin marks of active transcription.

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